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Observations on Preadipocytes and Their Distribution Patterns in Rat Adipose Tissue

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ABSTRACT Microscopic examination of adipocytes isolated from adult rat epididymal adipose tissue revealed numerous small cells (< 10 μm) morphologically similar to larger adipocytes. These small adipocytes appear identical to a new classification of adipose cells termed preadipocytes. Electron micrographs of these preadipocytes revealed examples of cells < 10 μm in diameter in various stages of maturation and lipid accumulation. The percent distribution pattern of these small adipocytes was not significantly altered by exercise although exercise shifted the distribution patterns of the larger cells (> 30 μm) toward a smaller mean cell size. The quantitative significance of preadipocytes is not established but these preliminary observations indicate that adipocytes < 10 μm in diameter may account for a numerically greater proportion of the total adipocytes observed in collagenase isolated preparations than heretofore recognized, although their contribution to total adipose mass is probably negligible.

The majority of studies on adipose tissue cellularity have utilized the osmium fixation-Coulter counter method of determining cell size and number (Hirsch and Gallian, '68). While this procedure has proven to be a rapid and reliable method for investigating alterations in adipose tissue cellular composition, adipocytes smaller than 25 μm diameter are not detected due to filter mesh size limitations (Stern and Greenwood, '74). The microscopic method of adipocyte sizing of Di Girolamo, Mendlinger and Fertig ('71), although more time consuming, permits the determination of cell diameters less than 25 μm as well as those cell sizes normally detected by the osmium-fixation method. Stiles, Francendese, and Masoro ('75), utilizing a modification of the microscopic cell sizing method of Di Girolamo et al. ('71), have recently reported the presence of cells from collagenase preparations of rat adipose tissue < 10 μm diameter that were morphologically similar to larger adipocytes. These investigators speculated that these previously unreported small cells may be preadipocytes that could mature as the "need arises."

We first noted the presence of what appeared to be very small cells in collagenase preparations of adipose tissue cell suspensions during our investigations of the influence of exercise training on adipose tissue cellularity (Askew and Hecker, '76). The surprisingly large number of these small cells stimulated us to characterize them further. We have confirmed and extended the observations of Stiles et al. ('75) by documenting the existence of preadipocytes, characterizing their morphology, and recording the relative frequency of occurrence of a complete range of adipocyte sizes in rat epididymal adipose tissue. In addition, the influence of exercise training, a treatment known to influence adipose tissue mean cell size (Askew, et al. '75), was evaluated in relationship to the adipocyte distribution patterns.

MATERIALS AND METHODS

Animals and training

Male Carworth CFN rats (Carworth, Portage, Michigan) 6½ weeks of age, weighing approximately 130 gm, were initially divided into two treatment groups designated trained (N = 14) and untrained (N = 9). The trained group was subjected to 12 weeks of exercise training by treadmill (Quinton Scientific, Seattle, Washington) running. At the end of 12 weeks, the trained rats were running for 120 min/day,

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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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8° grade at 29.5 M/min with 42.9 M/min sprints of 30 sec duration interspersed every 10 min of running time. An exercise program similar to this one has been shown to cause a significant reduction in adipose cell size with no change in cell number (Askew et al., '75). The untrained rats remained sedentary in their cages for the duration of the experiment. Both groups of rats were permitted to consume a lab stock diet (Purina Lab Chow, Ralston Purina Co., St. Louis, Missouri) ad libitum.

Fat cell isolation

At the end of the 12-week training program, (18½ weeks of age) rats were killed by decapitation and the left epididymal fat pad was removed and rinsed in 0.15 M KCl at room temperature. Fat cells were isolated by a modification (Therriault, Hubbard, and Melin, '69) of Rodbell's procedure (Rodbell, '64). Minced fat tissue was incubated for 90 minutes in Krebs-Ringer phosphate buffer (6 ml/gm), pH 7.4, prepared without calcium or glucose, and containing 4% bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri, Fraction V) and 3 mg/ml collagenase (Calbiochem, La Jolla, California, Type R). The cell digestion was accomplished in capped conical 60 ml polyethylene centrifuge tubes suspended horizontally and shaken at 150 revolutions/minute in a 37° gyrotory water bath. At the end of the 90-minute incubation period, cellular disaggregation was virtually complete. The cells were washed 3 times with 30 ml/wash Krebs-Ringer phosphate plus 4% bovine serum albumin buffer, pH 7.4. Following each wash and a 3-minute 300 × *g* centrifugation at room temperature, the cellular debris and infranatant was aspirated off with a siliconized dispopipette taking care to disturb the floating fat cells as little as possible. Approximately 7 ml of the 30 ml wash was left beneath the cells after each wash to minimize possible cell loss during the aspiration procedure. The washed cells from one pad were suspended in 30 ml buffer prior to microscopic examination. Histological examination of the isolated fat cells stained with methylene blue or a modified methylene blue stain (0.13 gm methylene blue, 0.02 gm azure II, 80 ml H_2O, 10 ml glycerol, and 10 ml methanol) revealed freely dispersed unilocular, spherical fat cells, free of stromal-vascular material. Lipid droplets were easily distinguished as lacking nuclei and peripheral cytoplasmic rims.

Light microscopy

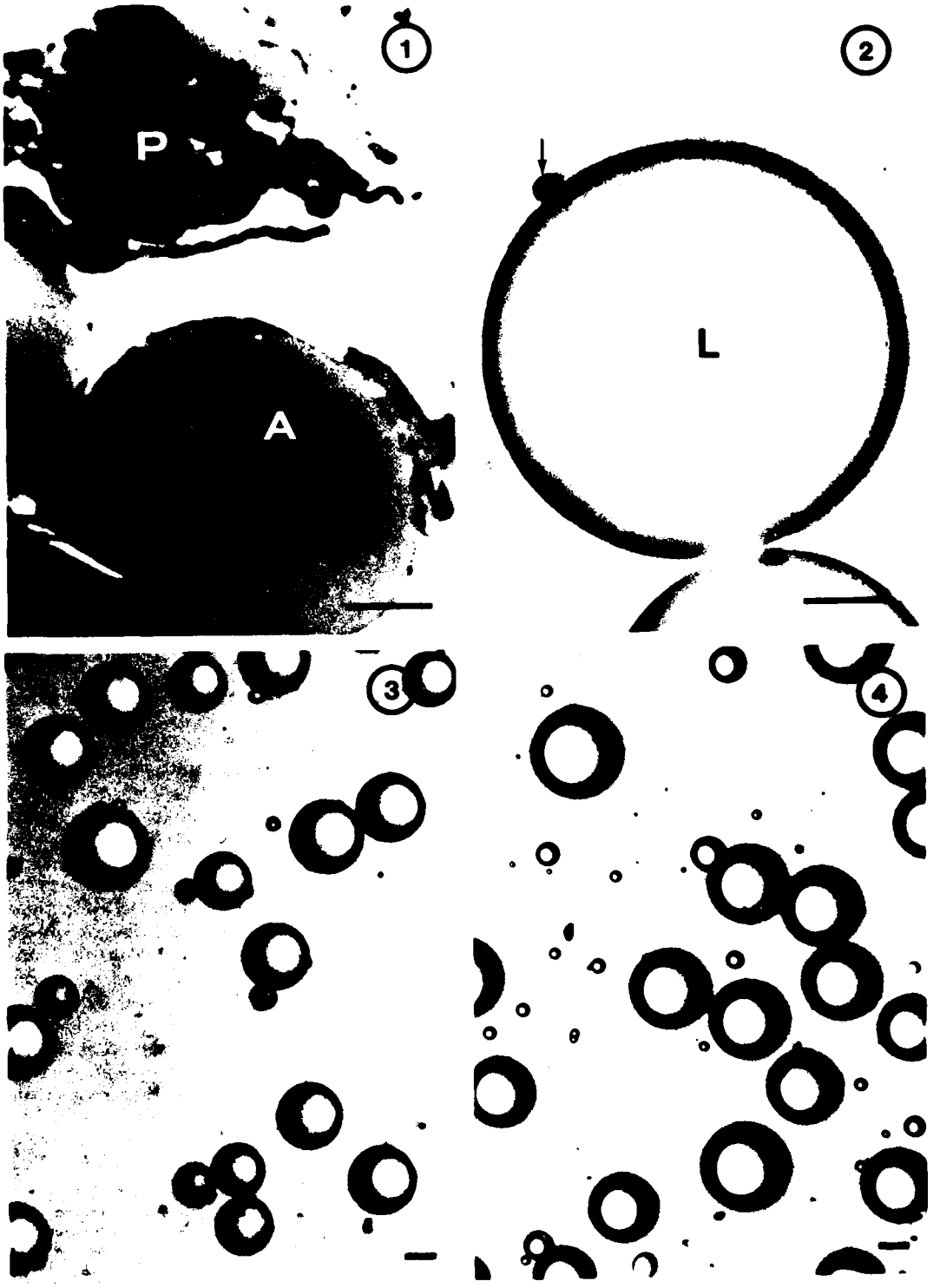
A photographic modification of the method of Di Girolamo et al. ('71) was used for direct microscopic determination of fat cell diameter. Each cell preparation was examined with the aid of a Leitz photomicroscope (Earnest Leitz, Wetzler, Germany). Six fields 150–200 total cells per rat) were photographed at a magnification of 100 ×. A calibrated micrometer slide (smallest division 10 μm) was photographed with each set of cells. The micrometer scale was used to measure the diameters of the photographed cells (cells were enlarged approximately 160 × on prints). This procedure permitted rapid processing of the cells while minimizing any tendency toward spontaneous cell rupture. Cells less than 10 μm in diameter were tentatively identified as preadipocytes by examining preparations stained with the modified methylene blue stain at a magnification of 450 × with the aid of a dual viewing binocular light microscope (A.O. Spencer, American Optical, Richmond, California). The percent recovery of fat pad triglyceride in the adipocyte preparations was approximately 82% and did not differ significantly between groups. Statistical analysis of

Abbreviations
P, preadipocytes
A, adipocyte
L, lipid
N, nucleus

Fig. 1. Photomicrograph of an osmium fixed section of intact epididymal adipose illustrating a "pocket" of preadipocytes (P) occurring in the midst of normal sized mature adipocytes (A). A 10 μm bar is superimposed on the photograph.

Fig. 2. A normal sized mature adipocyte approximately 110 μm in diameter. The arrow denotes the nucleus and the cytoplasmic rim can be seen surrounding the central lipid vacuole (L). A 25 μm bar is superimposed on the figure.

Fig. 3 and 4. Two representative adipocyte isolations following collagenase treatment of epididymal adipose tissue as described under "Materials and Methods." Note the numerous small spherical structures interspersed among the larger adipocytes. They are especially evident in Figure 4. These small structures were tentatively identified as preadipocytes. The focus in Figure 3 is on the large cells, while the focus in Figure 4 is on the small cells, accounting for the blurred periphery of the larger cells in Figure 4. A 25 μm bar has been superimposed on Figures 3 and 4.


1
2
P
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fat cell distribution patterns was accomplished by a one-way analysis of variance (Steele and Torrie, '60), level of significance $P < 0.05$. Values shown represent the mean ± SE.

Intact epididymal adipose tissue was examined by light microscopy to determine if small adipocyte-like cells, similar to those observed in collagenase preparations, could be detected *in situ*. An epididymal fat pad was removed from one trained and one untrained rat, sectioned into 4-mm-thick slices and fixed in 3% glutaraldehyde in 0.15 M Sorensen's phosphate buffer. The slices were processed by osmicating in 1% OsO_4, washing in phosphate buffer, dehydrating in ethanol, and embedding in Epon-Araldite. Blocks were cut to a depth of 0.5 mm to allow full-face sampling of the tissue, mounted and stained with methylene blue - azure II and 0.2% aqueous basic fuschin stain. Specimens were examined and photographed at 40 ×.

Electron microscopy

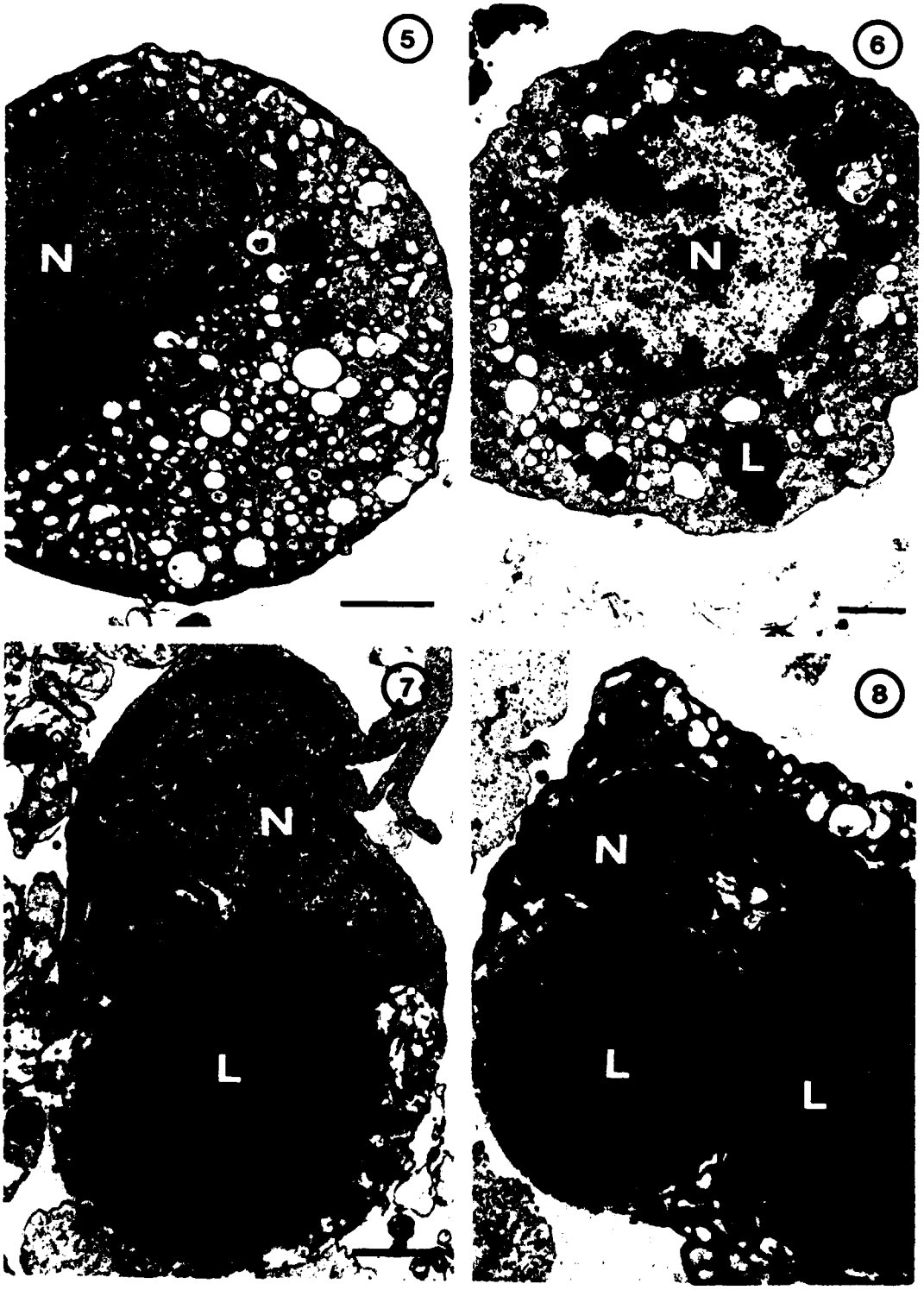
In addition to examination of adipocytes by light microscopy, adipocyte preparations from three rats were examined by electron microscopy. The cells were prepared as described previously. The isolated washed adipocyte suspension was fixed in 8% glutaraldehyde, rinsed in cacodylate buffer and osmicated in 1% OsO_4, and embedded in Epon as described by Stiles et al. ('75). Sectioning was accomplished with an LKB III ultra microtome. Sections were stained with uranyl acetate and lead citrate and photographed through a Hitachi model HS-8F-2 electron microscope at 50 KV.

RESULTS

There is apparently no consensus of opinion concerning the exact diameter which should serve as the dividing line between preadipocytes and mature adipocytes. Indeed, there exists no clear distinction at which point a cell becomes a preadipocyte. Björntorp et al. ('79) have proposed a classification system for adipose cells that includes a combination of morphological and functional characteristics. We have designated spherical cells of less than 10 μm diameter as preadipocytes to agree with previous usage of the term (Stiles et al., '75). Histological sections of epididymal adipose tissue were examined to determine if preadipocytes could be located *in situ* in intact adipose tissue. There did not appear to be a uniform distribution of preadipocytes among the larger mature adipocytes. When small cells, less than 10 μm diameter, were observed they appeared to be clustered in "pockets" among the larger cells. Such a "pocket" of what appeared to be preadipocytes among larger adipocytes is shown in Figure 1. Of the 28 blocks examined in this study, seven possessed clusters of small spherical structures resembling preadipocytes. These data on the relative frequency of occurrence of preadipocytes in intact adipose tissue should be regarded as tentative, due to the small sample number observed. Osmium penetration into our intact tissue slices were not always complete, preventing a more comprehensive assessment of the incidence of preadipocytes.

Numerous cell-like spherical objects smaller than 10 μm in diameter, were present in the collagenase preparations. Representative cell preparations were examined at higher magnification with the aid of a light microscope (Fig. 2, 3, and 4). The modified methylene blue stain gave good contrast to the cells, staining the nucleus and peripheral cytoplasmic rim darker than the central lipid vacuole. Lipid droplets existing in the preparations did not stain and were easily excluded. A normal sized adult adipocyte is shown in Figure 2. Two representative collagenase isolations of adipocytes are shown (Fig. 3 and 4). Note the small spherical structures less than 10 μm in diameter. These structures were tentatively identified as preadipocytes. Figure 4 contained relatively more preadipocytes than Figure 3, illustrating the variability encountered in isolating these cell types. Examination of the spherical objects less than 10 μm in diameter at 450 × magnification revealed cells possessing morphological characteristics similar to larger adipocytes. The cells were unilocular and appeared to have a central vacuole or lipid droplet enclosed in a rim of cytoplasm. Adipocyte preparations were examined further by electron microscopy to es-

Fig. 5, 6, 7, 8. Electron micrographs of preadipocytes in several stages of development. These cells were obtained from epidydimal adipose tissue of rats 20 weeks of age, weighing approximately 400 gm. Numerous preadipocytes in various stages of development were found. Four typical structures are shown. Fig. 5. Preadipocyte without appreciable lipid vacuoles; Fig. 6. Preadipocyte with several small lipid vacoules; Fig. 7. Preadipocyte with one large central lipid vacuole, and Fig. 8. Preadipocyte with two lipid vacuoles. The nuclei in these figures are labeled N and the lipid vacuoles are labeled L. A 1 μm bar is superimposed on each electron micrograph.


5
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L

tablish morphologies of the preadipocytes. Typical structures identified as preadipocytes are shown in Figures 5-8. We have selected four preadipocytes that demonstrate a developmental progression in lipid accumulation. Figure 5 shows a cell with numerous vacuoles but little evidence of lipid storage. Figure 6 illustrates a preadipocyte in the early stages of lipid accumulation. Figure 7 shows a preadipocyte that has a well-defined central lipid vacuole and is morphologically similar to larger fat cells except for the smaller degree of lipid accumulation. Figure 8 shows an adipocyte possessing two lipid vacuoles. The multi-vacuole feature displayed by small adipocytes was not observed in mature adipocytes, suggesting that at some point in their development the lipid vacuoles fuse into one large storage vacuole.

A curious arrangement of mitochondria around the periphery of the central lipid vacuole was observed in one preadipocyte (not shown). This arrangement was not observed in other preadipocytes examined in the course of this study.

The relative occurrence of preadipocytes in adipose tissue of exercise-trained and sedentary rats was examined to see if exercise training prevented or delayed the transition of these small adipose cells into larger adipocytes. As noted previously (Askew et al., '75), untrained sedentary rats weighed significantly more than trained rats (370 ± 12 gm vs. 283 ± 6 gm) and possessed significantly larger epididymal fat pads (2.4 ± 0.2 gm vs. 1.1 ± 0.1 gm). The distribution of adipose tissue cell sizes ranging from < 10 μm to > 100 μm in trained and untrained rats is shown in figure 9. Cell numbers in the < 10 μm class should be viewed as tentative due to limitations of light microscopy to positively identify objects in this size range. The bimodal distribution pattern shown in figure 9 is in contrast to the more normal distribution pattern observed when only cell sizes greater than 30 μm are plotted. These observations are in agreement with those of Mersmann, Goodman, and Brown ('75) who noted biphasic adipocyte distribution patterns in swine adipose tissue, and Kirtland, Gurr, and Saville ('75) who documented a distinct population of small adipocytes in adipose depots of guinea pigs. As shown previously (Askew et al., '75), training shifted the median of the cells with diameters > 30 μm toward smaller cell sizes. This was accomplished primarily by reducing the number of cells in the > 70 μm range and correspondingly increasing the frequency of occurrence of cells in the 30-70 μm size range. There were no significant differences in the distribution of cell diameters in the < 30 μm groups. Trained rats tended to have slightly more of the < 10 μm cells compared to the untrained rats; however, this difference was not significant (P > 0.05).

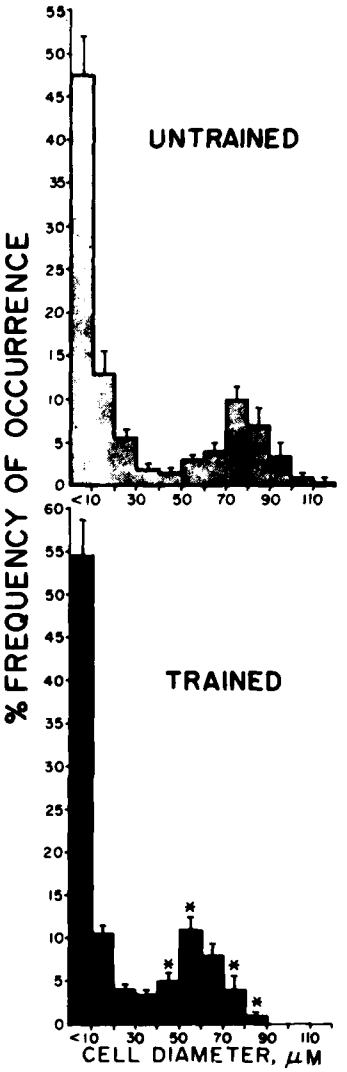

Fig. 9. Adipocyte distribution pattern in epididymal adipose tissue from untrained sedentary and exercise-trained rats. Methods of cell isolation and sizing are described under "Materials and Methods." The bar values represent the mean ± SE for each cell size classification. N = 9 for untrained rats and 14 for trained rats. Significant differences in percent frequency between trained and untrained rats for each 10 μm division is denoted by an asterisk above the group that is significantly different, P < 0.05.

DISCUSSION

Van and Roncari ('77) have recently demonstrated that adult rat adipose tissue contains

cells possessing the potential to proliferate and acquire characteristics similar to adipocytes. Other studies suggest that small "pockets" of immature adipocyte cells exist in fat depots (Kirtland et al., '75; Ashwell, Priest, and Sowter, '75) and may represent loci for new fat cell formation (Ashwell et al., '75). The transition from preadipocytes to larger adipose cells may be at least partially controlled by the activity of the enzyme lipoprotein lipase (Hietanen and Greenwood, '77). Exercise training initiated prior to the fourth postnatal week in rats has been shown to decrease both cell size and cell number (Oscai et al. '72), while exercise training initiated after this point exerts an influence on cell size, but not cell number (Askew and Hecker, '76). In the present study exercise training, although effective in shifting adipocyte distribution patterns toward smaller cells, did not appear to have any significant effect on the percent frequency of occurrence of small cells $< 10\ \mu m$ in diameter. This raises the question of the physiological role of this population of adipocytes. Stiles et al. ('75) have suggested that small adipocytes may function as a pool of preadipocytes which mature as the need arises. Such a concept is only partially supported by the results of this study, since one might have possessed fewer preadipocytes (due to greater filling with lipid and subsequent transformation to the larger cell size) and the trained rats would have predicted that the untrained rats would have more preadipocytes (due to less filling with lipid because of a diversion of rat from storage to oxidation for energy). A greater intensity of exercise or exercise imposed at an earlier developmental stage might accentuate these differences; however, this point remains to be determined.

It is apparent that none of the current methods for determination of adipose cell size and cell number will detect preadipocytes before they achieve a certain critical buoyancy due to lipid accumulation (Stern and Greenwood, '74). The $< 30\ \mu m$ diameter cell distribution patterns presented in this paper should not be regarded as quantitative since the washing procedure used to isolate the cells undoubtedly results in the aspiration and loss of small, less buoyant cells, especially if the floating cell layer is mechanically disturbed during aspiration. Indeed, the 50% frequency figure for $< 10\ \mu m$ diameter cells arrived at in this report may only represent a portion of the potentially detectable preadipocytes. Therefore, a note of caution is advised in interpreting these results. Identification and quantification of small adipose cells is a tentative procedure at best. It is impossible to state with certainty that all of the cells classified as preadipocytic in the light microscopy counting portion of this experiment are preadipocytes. Small lipid droplets with adhering cytoplasmic fragments would be difficult to exclude and might be included as a source of error in these cell counts.

The results of this study confirm the presence of small adipocyte-like structures reported previously (Stiles et al., '75) in rat adipose tissue and suggest that if these structures are indeed preadipocytes, they account for a much larger percentage of the total cell number in adipose tissue than previously recognized. Since the amount of triglyceride contained in these small adipocytes is probably not significant in relation to the total mass of adipocyte triglyceride contained in the total adipose depot (Stiles et al., '75), their metabolic significance remains to be established. A recent report by Rubin, Lai, and Rosen ('77) suggests that preadipocytes established in cell culture lack the degree of hormone responsiveness associated with differentiated adipocytes. The exact size or stage of differentiation when preadipocytes become metabolically active has not been established. Such a determination would be of practical significance to investigators utilizing isolated adipocytes in metabolic studies. Björntorp et al. ('78) have demonstrated that rat adipose cells in the preadipocyte phase of maturation possess well-developed lipoprotein lipase activity but relatively low lipolytic activity. Until such time as definitive studies on the contribution of preadipocytes to overall adipose tissue metabolic activity become available, it is important to recognize the existence of this type of cell in collagenase preparations of adipose tissue cells.

The unique orientation of mitochondria around the central lipid vacoule of one preadipocyte observed suggests that some event concerned with energy metabolism may be occurring that is facilitated by the close juxtaposition of mitochondria and the lipid vacuole, possibly triglyceride formation. Napolitano ('63) studied modifications of the ultrastructure of adipose cells in the process of accumulating lipid and reported that mitochondria were not found in close association with fat droplets nor did they appear in greater numbers in areas of the cytoplasm where lipid was being deposited. However, certain observations suggest that the pattern of mitochondrial association with the lipid droplet that we observed would be consistent with transfer of newly synthesized glyceride from the mitochondria to the storage pool (Hollenberg, Angel, and Steiner, '70). Usuku,

Iyama, and Ohzono ('78), in contrast with the observations of Napolitano ('63), reported that developing adipose tissue has large and small lipid droplets surrounded by smooth endoplasmic reticulum that is in close contact with mitochondria. More definitive studies are needed to determine if mitochondria orientation is related to preadipocyte maturation.

Our study suggests that small cells exist in adipose tissue of adult rats that possess morphologies of preadipocytes. These immature adipocytes may be more numerous in adipose tissue of adult rats than previously recognized.

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